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Stabilization of a recombinant human epidermal growth factor parenteral formulation through freeze-drying

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ABSTRACT

Development studies were performed to design a pharmaceutical composition that allows the stabilization of a parenteral rhEGF formulation in a lyophilized dosage form. Unannealed and annealed drying protocols were tested for excipients screening. Freeze-dry microscopy was used as criterion for excipients and formulation selection; as well as to define freeze-drying parameters. Excipients screening were evaluated through their effect on freeze-drying recovery and dried product stability at 50 °C by using a comprehensive set of analytical techniques assessing the chemical stability, protein conformation and bioactivity. The highest stability of rhEGF during freeze-drying was achieved by the addition of sucrose or trehalose. After storing the dried product at 50 °C, the highest stability was achieved by the addition of dextran, sucrose, trehalose or raffinose. The selected formulation mixture of sucrose and dextran could prevent protein degradation during the freeze-drying and delivery processes. The degradation rate assessed by RP-HPLC could decrease 100 times at 37 °C and 70 times at 50 °C in dried with respect to aqueous formulation. These results indicate that the freeze-dried formulation represents an appropriate technical solution for stabilizing rhEGF.

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1. Introduction

About 347 million people are currently affected by diabetes mellitus (DM) [1]. World Health Organization (WHO) estimated that diabetes will be the 7th highest cause of death in 2030 [2]. Diabetic patients have a reduced capacity to carry out tissue repair processes, and are more susceptible to chronic wounds, such as leg ulcers and diabetic foot ulcers (DFU), that frequently cause hospitalizations, amputation and morbidity [3]. An estimated 15% of patients with DM develop foot ulcers. Ten-year mortality rates are increased to 50% in patients with DFU compared to patients without ulceration [4]. A wide range of medical interventions are used for DFU management and new therapies are emerging to promote wound healing, including topically applied growth factors [5], skin substitutes [6,7], and others that have shown efficacy in relatively small, pure neuropathic, non-complicated ulcers [8]. Amputation is still a foreseeable outcome in cases with large, advanced DFU, even more if ischemia is present.

The epidermal growth factor (EGF) induces mitogenic, motogenic, and cyto-protective actions that are instrumental for the healing process [9]. The availability of the growth factor at the deeper layers of the wound is an important issue to obtain an adequate therapeutic effect. This can be a limitation for topical formulations because the active agent bioavailability is affected by necrotic tissue, sepsis, inflammation and wound proteases [10]. Significantly, the addition of metalloproteinase inhibitors can revert the substantial degradation of exogenous EGF and its receptor in chronic ulcers, implying that EGF is susceptible to the proteolytic environment of such wounds [11]. The local infiltration of EGF into the wound base and edges possibly reduces its degradation following topical application and contact with wound

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exudates. Recently, a new first-in-class treatment based on local (intralesional) instillation of recombinant human EGF (rhEGF) to heal advanced DFU has been developed. The results of this intervention can be considered clinically relevant because they offer an alternative treatment for advanced ulcers (Wagner's grade 3 or 4, mostly above 20 cm^2 wide, including ischemic) at higher risk of amputation [12].

To use the rhEGF as local infiltrations drug for DFU treatment, it is necessary to develop a safe and effective formulation by stabilizing the protein during production and storage. EGF is a small protein, molecular weight 6230 g/mol, containing three internal disulfide bridges. Previous studies have identified the Asn-1 deamidation, the conversion of Asp-11 to a stable Asp-succinimide and Met-21 oxidation as critical factors that hindered rhEGF stabilization in a liquid formulation [13]. The modification of both Asn and Asp residues (Asx) is one of the major chemical degradative pathways for proteins during manufacturing and storage. Although the reactivity of Asx residues is highly reduced in proteins formulated as lyophilized powders in some lyophilized formulations, it could still be significant enough to compromise protein stability [14]. Conceivably, shelf life of rhEGF in dried state could be increased due to absence of water and dissolved oxygen; thereby circumventing protein hydrolysis and oxidation.

Since rhEGF is very labile in aqueous systems, it is highly relevant to develop and characterize a successful lyophilized dosage form [13]. Accordingly, in this work, a screening study was performed to identify the excipients which mitigate the protein degradation during freeze-drying and storage at high temperature. Two drying protocols, unannealed and annealed, were tested for initial screening. Freeze-drying microscopy was used as criterion for excipients and formulation selection; as well as to define freeze-drying parameters. The effects of the sucrose to dextran ratio on rhEGF stability during the freeze-drying process, and storage of the dried formulation at high temperature, were also determined. In addition, protein stability was monitored using a comprehensive set of analytical techniques assessing the formation of aggregates, protein conformation and biological activity. The properties of freeze-dried cake, namely the reconstitution performance and the residual moisture content, and the stability of the rhEGF formulation at high temperatures in liquid and dried states were analyzed.

2. Materials and methods

2.1. Materials

A mixture of rhEGF1-51 and rhEGF1-52 expressed in *Saccharomyces cerevisiae* was supplied as concentrated bulk solution (CIGB, Havana, Cuba). Chemicals were of analytical grade and the excipients met the European Pharmacopoeia standards (EP). Dextran with molecular weights of 37,000–43,000, indicated as dextran, was used (AppliChem GmbH, Darmstadt, Germany). Neutral clear borosilicate glass type I hydrolytic quality vials (Nuova OMPI, Piombino Dese, Italy), bromobutyl type gray siliconized freeze-drying rubber stoppers and flip-off aluminum seals covered with polypropylene plastic cap (Helvoet Pharma, Alken, Belgium) were used. Water for injection (WFI) and normal saline solution (9.0 g/L NaCl; NSS) (Quimefa, Havana, Cuba), as well as disposable 27-gauge × 0.5-inch needle (VWR Scientific, San Francisco, CA, USA) coupled to a 5-mL silicone oil free disposable syringe (Fisher Scientific, Pittsburgh, PA, USA) were used in the injection simulation studies.

Table 2
Effect of dextran/sucrose ratio on T_{oc} and lyophilized cake attributes of rhEGF formulations.

Weight fraction of dextran[a] (w/w)	T_{oc} (°C)	Appearance of cake	Moisture content (%, w/w)	Reconstitution time (s)	Absorbance 350 nm
0	−35.3 ± 0.4	Partial collapse	3.3 ± 0.3	10 ± 2	0.005 ± 0.002
0.25	−30.8 ± 0.5	Noncollapsed	3.1 ± 0.3	13 ± 3	0.005 ± 0.002
0.5	−26.0 ± 1.4	Noncollapsed	3.0 ± 0.2	14 ± 2	0.006 ± 0.001
0.75	−19.6 ± 0.9	Noncollapsed	3.6 ± 0.3	16 ± 2	0.007 ± 0.002
1.0	−14.1 ± 1.2	Noncollapsed	4.2 ± 0.3	18 ± 3	0.008 ± 0.003

[a] Weight fraction of dextran = (weight of dextran)/(weight of dextran + weight of sucrose).

2.2. Preparation of rhEGF formulations

Phosphate buffer was chosen since it stabilizes rhEGF in the presence of many excipients during the freeze and thawing process [13]. Furthermore, sodium phosphate buffer at 10 mM was selected to minimize pH shift during freezing and avoid an unnecessary decrease in maximal freeze concentrate temperature, Tg', of protein formulation [15]. For the current study, rhEGF excipients previously shown to be incompatible, after solution stressed at high temperature and multiple freezes and thawing cycles were discarded [13]. rhEGF at 75 μg/mL formulations were prepared with 2% w/v excipients or excipients mixtures in 10 mM phosphate buffer, pH 7.0. The solutions were filtered using 0.22 μm filters (Millipore, Bedford, Massachusetts, USA) prior to freeze-drying. The individual excipients tested are shown in Table 1, and the different sucrose-dextran mixtures tested in Table 2 as weight fraction of dextran (w/w). As a negative control, a rhEGF solution of 75 μg/mL (without any excipients) was freeze-dried at the same settings as the formulations.

Table 1
List of lyoprotectants used in this study with corresponding onset of collapse temperature (T_{oc}) and lyophilized cake attributes.

Excipient	T_{oc} (°C)	Cycle	Appearance	Moisture content (%, w/w)	Reconstitution time (s)	Absorbance 350 nm
Buffer (sodium phosphate)	−49.5 ± 0.5	Unannealed	White, shrunken	3.7 ± 1.4	6 ± 1	0.006 ± 0.001
Sucrose	−35.3 ± 0.4	Unannealed	White, slightly shrunken	3.1 ± 0.3	9 ± 2	0.005 ± 0.003
Trehalose	−32.4 ± 1.2	Unannealed	White, slightly shrunken	3.4 ± 0.6	11 ± 3	0.004 ± 0.004
Raffinose	−27.7 ± 0.9	Unannealed	White, non-shrunken	3.2 ± 0.4	13 ± 3	0.004 ± 0.002
Dextran 40	−14.1 ± 1.2	Unannealed	White, non-shrunken	3.9 ± 0.5	16 ± 2	0.009 ± 0.004
Sorbitol	−45.9 ± 1.7	Unannealed	White, shrunken	3.7 ± 0.5	29 ± 2	0.003 ± 0.002
Mannitol	−29.6 ± 0.8	Unannealed	White, non-shrunken	3.1 ± 0.3	9 ± 1	0.003 ± 0.001
		Annealed	White, non-shrunken	2.6 ± 0.3	10 ± 3	0.003 ± 0.001
Glycine	−9.7 ± 0.5	Unannealed	White, non-shrunken	2.8 ± 0.5	11 ± 1	0.005 ± 0.004
		Annealed	White, non-shrunken	2.3 ± 0.3	16 ± 2	0.004 ± 0.002

Sugar and polymer: sucrose, trehalose, raffinose and dextran-40.
Polyol: mannitol, sorbitol.
Amino acid: glycine.

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